Synergistic Attraction of *Aedes aegypti* (L.) to Binary Blends of L-Lactic Acid and Acetone, Dichloromethane, or Dimethyl Disulfide

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ABSTRACT Kairomones produced by humans provide female anthropophilic mosquitoes with vital cues used in host-seeking for a blood meal. These chemicals are emanated primarily by the skin and provide the mosquitoes a means to orient themselves to humans at a relatively close range. Chemical studies of these emanations have provided new ideas for the formulation of attractant blends. We report mosquito attraction responses for three binary blends and their separate components. The blends are comprised of L-lactic acid plus either acetone, dichloromethane, or dimethyl disulfide. At the emission rates used in our bioassays, these blends synergistically attract laboratory-reared female *Aedes aegypti*. Carbon dioxide is not a necessary component to yield high levels of attraction with these blends. It is postulated that at least one of these synergistic blends (L-lactic acid and acetone) produces mosquito attraction behavior similar to L-lactic acid and CO_2.

KEY WORDS kairomones, behavioral bioassay, mosquito attractants

HOST-SEEKING MOSQUITOES are attracted by various cues, such as vision, moisture, heat, carbon dioxide, and host emanations (McIver 1968, Price et al. 1979, Gillies 1980, Takken 1991, Takken and Knols 1999). Until recently, few reports exist of mosquitoes being attracted to host (human) odors independently of carbon dioxide (Schreck et al. 1981). Carbon dioxide activates and results in upwind flight of mosquitoes (Schreck and James 1968, Gillies 1980, Geier et al. 1999). Experiments involving removal of carbon dioxide from exhaled breath showed reduction in mosquito attraction to a host; however, it is not certain that carbon dioxide was the only volatile compound removed in such a study (Gillies 1980). Carbon dioxide and acetone are major components of breath, and breath has been found to attract *Aedes aegypti* (L.) in the olfactometer used for these studies (U.R.B., unpublished data).

The human body attracts mosquitoes very efficiently and respiration of carbon dioxide through the skin is essentially negligible (Frame et al. 1972, King et al. 1978). Therefore, other human-produced volatiles are suspected to be contributing to the activation in place of carbon dioxide. Chemical studies have shown that of the moderately volatile emanations, carboxylic acids and lactic acid are present in the highest amounts (Bernier et al. 2000). Recent work has demonstrated that low emission or release rates of these acids are attractive to mosquitoes (Bosch et al. 2000). Microscale purge and trap gas chromatography/mass spectrometry (GC/MS) studies of the more volatile components revealed that, by this method, acetone was observed to be the most abundant component emanating from the human hand (M.M.B., unpublished data).

L-Lactic acid is a well known attractant for *Ae. aegypti* and it also plays a significant role in host-finding by *Anopheles gambiae* Giles (Acree et al. 1968, Dekker et al. 2002). From observations in the olfactometer, L-lactic acid attracts female *Ae. aegypti* mosquitoes, but the attraction is not great. Carbon dioxide activates mosquitoes to take flight, and when combined with lactic acid, the binary combination provides formidable attraction to the source of the odors (Smith et al. 1970). The drawback to the use of carbon dioxide in the field is that it can be somewhat burdensome, considering it needs to be either delivered via a tank, dry ice, or produced by combustion. Therefore, discovery of suitable less volatile replacements for CO_2 would be highly beneficial in terms of both portability and potentially a reduction in cost of baits.

To date, the search for attractants has been aimed at discovering a compound or blends of compounds that attract specific species. Such a mixture would presumably involve synergistic action of blend components. Previous work has demonstrated that certain combinations of chemicals, such as 1-octen-3-ol and CO_2, synergistically attract some species more than others (Takken and Kline 1989; Takken 1991; Kline et al. 1990, 1991a, b).

We report chemicals used alone or in combination with L-lactic acid that are attractive to mosquitoes.

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The blends produce high levels of attraction without employing carbon dioxide. Dichloromethane is halogenated and suspected not to be endogenous to humans; however, dichloromethane and acetone were tested against *Ae. aegypti* previously (USDA 1964). Acetone was too volatile to be detected by previous methods employing glass beads subjected to thermal desorption and cryo-focused GC/MS analysis; however, dimethyl disulfide was detected by this method (Bernier et al. 2000, 2002).

Materials and Methods

Chemicals. Acetone [67–64-1] 99.5+% A.C.S. reagent grade, dichloromethane [75–09-2] 99.6% A.C.S. spectrophotometric grade, and dimethyl disulfide [624–92-0] 99% were purchased from Aldrich (Milwaukee, WI). L-(+)-lactic acid [79–33-4] >99% was purchased from Fluka Chemical Company (Milwaukee, WI). A 2 μg/μL L-lactic acid stock solution was prepared in reagent grade methanol UltimAR [67–56-1] 99.9+% (Mallinckrodt Chemical, Phillipsburg, NJ).

Procedure. Bioassays were conducted in a triple-cage dual-port olfactometer (Posey et al. 1998). Each cage allows for a single experiment to be conducted. The mosquitoes can select to either remain in the cage or fly upwind and be captured in one of two ports containing a treatment. The lactic acid treatment consisted of a 100 μL aliquot of methanolic L-lactic acid plated out onto a vial cap of 15 mm i.d. (internal diameter) by 9.5-mm height, with a total cap volume of 1.4 mL. The solution was allowed to dry for at least three minutes to remove the methanol solvent. Methanol is employed as the solvent because it is not attractive to *Ae. aegypti* in these bioassays (Bernier et al. 2001). Acetone, dimethyl disulfide, and dichloromethane were placed in a vial cap (9 mm i.d. $\times$ 9 mm height, total volume 400 μL). Each of these compounds was filled to the top of the vial and allowed to evaporate freely in the olfactometer port during the experiment; thus, the surface area of the cap and the volatility of the compound played a significant role in the emission rate. The average emission rate was calculated by measuring the loss in mass of each of the samples, in triplicate, for a 3-min test under identical conditions (27 $\pm$ 1°C, 60 $\pm$ 2% RH, and air flow of 28 $\pm$ 1 cm/s) to those used throughout all experiments. The rates were 32.9 $\pm$ 0.2 mg/min for acetone, 8.9 $\pm$ 0.1 mg/min for dimethyl disulfide, and 72.3 $\pm$ 0.2 mg/min for dichloromethane.

Approximately 75 female *Ae. aegypti* mosquitoes (6–8 d old) were selected for each test cage by from a hand draw box using the method of Posey and Schreck (1981). A specifically designed trap was used to collect the mosquitoes and load them into each of the cages. Once all of the cages were loaded, the mosquitoes were allowed to acclimate for approximately one hour. During this acclimation period, a small flow of air was passed through the ports into each of the olfactometer cages. Treatments were randomized with respect to order, ports, time of day and cage within a complete block design. A total of 18 replicate tests were made for each treatment. Each test consisted of measuring the percentage of mosquitoes initially in the cage that were attracted to the port containing the treatment. The control port contained identical vial caps to that in the treatment port, and both the treatment and control ports had the same flow rate of air passing through them. Data were analyzed by SAS using General Linear Models (proc GLM), Duncan's Multiple Range Test, and proc TTEST (SAS Institute 1988).

Table 1. Average attraction (%) of female *A. aegypti* mosquitoes to single compounds and blends

Treatment	n	Mean $\pm$ SE (%)
L-lactic acid + dichloromethane	18	80.2 $\pm$ 2.4a
L-lactic acid + dimethyl disulfide	18	78.4 $\pm$ 3.4a
L-lactic acid + acetone	18	77.9 $\pm$ 2.1a
Dichloromethane	18	39.4 $\pm$ 4.1b
Acetone	18	26.2 $\pm$ 3.8c
Dimethyl disulfide	18	25.3 $\pm$ 2.1c
L-lactic acid	18	20.5 $\pm$ 3.2c

Means (% of mosquitoes trapped) followed by the same letter are not significantly different using Duncan's Multiple Range test (α = 0.05, df = 102) of data analyzed using General Linear Models procedure ($P > 0.0001$, $F_{23,102} = 22.30$). Each replicate consists of an attraction response of approximately 75 female mosquitoes.

Results and Discussion

No more than 4% of the mosquitoes were attracted to the control ports throughout all tests; usually, no mosquitoes were captured in control ports. Significant attraction to control ports indicates contamination of the port with an attractive substance. The mosquito attraction to each compound and to the three binary blends are listed in Table 1. There was no statistically significant difference between the mosquito attraction responses to the three binary blends; however, the combined response of L-lactic acid plus the activator component was greater than the respective activators above a 99.9% confidence level in all cases. In the experiments reported here, these activators are compounds which typically result in 50–100% of the mosquitoes taking to flight in a test cage. This is activation regardless of attraction or orientation toward the attractive source (Acree et al. 1968). The single activators, except for dichloromethane, did not have statistically different attraction from each other.

The levels of attraction observed for these blends are equivalent to or slightly greater than those seen when a human hand is tested in the olfactometer port. From separate studies with human arm and hand odors for three different human subjects, a range of 67–78% average attraction was observed (Bernier et al. 2001). It is important to note that this does not mean that the combination can, in direct competition, attract *Ae. aegypti* better than emanations from humans can.

Dichloromethane is the most volatile of the three synergists tested, while dimethyl disulfide is the least volatile. It should be pointed out that the emission rates, as reported in the materials and methods section, are only applicable to the conditions of these exper-

Table 2. Comparison of the percent attraction of the summed individual compound responses to that of the percent attraction to blends of those individual compounds

Treatment	Individuals[a] Mean ± SE (%)	Blend Mean ± SE (%)	t
L-lactic acid + dichloromethane[b]	59.9 ± 2.4	80.2 ± 2.4	3.22*
L-lactic acid + dimethyl disulfide[c]	45.8 ± 4.0	78.4 ± 3.4	6.21*
L-lactic acid + acetone[d]	46.7 ± 2.1	77.9 ± 2.1	5.70*

[a] These are the summed responses of the individual (single compound) treatments. The standard errors reported here are output variables of the SAS proc TTEST. The proc TTEST is a test of means and estimates t for unequal variances, otherwise it assumes that they are equal.
[b] The difference between the sum of individual responses for L-lactic acid and dichloromethane was significantly different than the response for the combined blend (proc TTEST; df = 34, $P = 0.0028$).
[c] The difference between the sum of individual responses for L-lactic acid and dimethyl disulfide was significantly different than the response for the combined blend (proc TTEST; df = 33, $P \geq 0.0001$). For the case, the variances were unequal.
[d] The difference between the sum of individual responses for L-lactic acid and acetone was significantly different than the response for the combined blend (proc TTEST; df = 34; $P > 0.0001$).

iments and the containers used to deliver the compounds in the olfactometer port.

The sums of individual compound attraction responses are compared with the attraction responses for each blend in Table 2. The definition of synergism is that the response to the combined mixture is greater than the sum of responses to individual compounds (Takken 1991, Geier et al. 1996). For these experiments, under the conditions described herein, all three blends were indeed synergistic, at a confidence of 99.7% or greater.

The response of these chemicals, blends, and many others tested by Bernier et al. (2001) has resulted in the formulation of a hypothesis as to how they interact. So far, we have chosen to use two terms to describe the mode of action. These are base attractant and activator. A base attractant can be described as a compound that causes minimal excitation in the mosquitoes but does results in attraction of the mosquitoes that do become activated to flight. Generally, if only a few mosquitoes take flight, a good base attractant will cause most of these to be collected in the port trap containing the treatment. L-lactic acid is the model base attractant for *Ae. aegypti* in this system. Activators, such as CO_2, cause high excitation but they typically yield low attraction. It has been documented that CO_2 alone in a clean port will attract virtually none of the test mosquitoes (Acree et al. 1968, Schreck and James 1968). What we observe in our olfactometer is that nearly all of the mosquitoes will be activated to flight, but only a few may be able to orient and fly into the trap where the emission of CO_2 is being released. The definition of these classes is somewhat ambiguous because activators still attract and base attractants can and do activate. This model of chemical classes based on how they impact mosquito attraction behavior is currently changing as more information is obtained.

In addition to acetone having been examined as a mosquito attractant alone, in combination with CO_2, it did not significantly increase catches over CO_2 alone for *An. gambiae* (Takken et al. 1997). It appears to us that acetone and CO_2 play a similar role in the host-location process. In preliminary experiments, the addition of CO_2 to acetone does not appear to increase the attraction efficiency, as either one does when combined with L-lactic acid (U.R.B., unpublished data). Current data from a separate set of tests show that dimethyl disulfide can be used in place of acetone but actually functions in a manner different from that of CO_2 and acetone. However, we still do not have a thorough understanding of the role of this disulfide in the overall attraction process.

Acetone appears to produce visually the same excitation effect as carbon dioxide, and in combination with L-lactic acid, it does attract at a level greater than or equal to L-lactic acid plus CO_2 (Bernier et al. 2001). Acetone results in mosquito orientation toward the port of the olfactometer that contains it. In our olfactometer, we can observe the mosquitoes orient themselves below the airstream containing acetone, whereas they tend to fly above it when L-lactic acid is present and acetone is absent. Many times, the mosquito attraction to L-lactic acid and acetone is greater than the percentage attracted to the human hand in noncompetitive tests. This implies that the efficiency of collection is greater for the chemical mixture; however, it does not imply that in direct combination, lactic acid and acetone is more attractive than the human hand. The results may indicate that classes of chemicals play a factor in mosquito attraction and that L-lactic acid plays a vital role, for this mosquito, as a synergist to many human produced odors. We believe that acetone simply takes on the same role as carbon dioxide for this species as it nears the host, and that it becomes a more efficient activator only at close range to the host.

Carbon dioxide is a nonspecific general kairomone used for host-seeking by many insect species, and high release rates of acetone may also be suitable as a substitute for attraction of *Ae. aegypti.* It is quite possible that the similarity in behavior is because of structural similarities in attractant molecules. Acetone contains a carbonyl functionality which is slightly more sterically hindered than the oxygens bonded to the carbon atom in the CO_2 molecule. The basis behind the attraction of mosquitoes to dichloromethane and dimethyl disulfide, and how these relate to other activators is still unclear.

All blends elicit probing from mosquitoes when in the olfactometer port trap, which is separated by a screen from the vial containing the chemical blends. The probing response appears to be dependent upon the amount of chemicals released, i.e., high levels appear to influence the mosquitoes such that they believe that they have reached the host. The collection efficiency is also somewhat dependent upon the amount of components released (Bernier et al. 2001). Further studies will investigate the optimum ratios of these components as well as evaluate the combination of other compounds with these two attractants.

Binary blends were chosen because of their simplicity and because of failures from earlier attempts with much more complex blends involving up to 30 compounds. There are chemicals that when added to highly attractive blends will depress the attraction and in some cases, completely inhibit it. These chemicals, which we prefer to call inhibitors rather than repellents, are currently being examined to better understand the overall factors involved with host-location using odors.

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